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High affinity interactions of Pb²⁺ with Synaptotagmin I

Sachin Katti¹, Bin Her¹, Atul K. Srivastava¹, Alexander B. Taylor², Steve W. Lockless³, and Tatyana I. Igumenova*¹

¹Department of Biochemistry and Biophysics, Texas A&M University, 300 Olsen Boulevard, College Station, TX 77843, United States

²Department of Biochemistry and Structural Biology and the X-ray Crystallography Core Laboratory, Institutional Research Cores, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, United States

³Department of Biology, Texas A&M University, 3258 TAMU, TX 77843, United States

Abstract

Lead (Pb) is a potent neurotoxin that disrupts synaptic neurotransmission. We report that Synaptotagmin I (SytI), a key regulator of Ca²⁺-evoked neurotransmitter release, has two high-affinity Pb²⁺ binding sites that belong to its cytosolic C2A and C2B domains. The crystal structures of Pb²⁺-complexed C2 domains revealed that protein-bound Pb²⁺ ions have holodirected coordination geometries and all-oxygen coordination spheres. The on-rate constants of Pb²⁺ binding to the C2 domains of SytI are comparable to those of Ca²⁺ and are diffusion-limited. In contrast, the off-rate constants are at least two orders of magnitude smaller, indicating that Pb²⁺ can serve as both a thermodynamic and kinetic trap for the C2 domains. We demonstrate, using NMR spectroscopy, that population of these sites by Pb²⁺ ions inhibits further Ca²⁺ binding despite the existing coordination vacancies. Our work offers a unique insight into the bioinorganic chemistry of Pb(II) and suggests a mechanism by which low concentrations of Pb²⁺ ions can interfere with the Ca²⁺-dependent function of SytI in the cell.

Significance to Metalloomics—Several signaling proteins that have been identified as molecular targets of Pb²⁺ contain C2 domains. C2 domains are Ca²⁺-dependent peripheral membrane modules that specifically bind to anionic phospholipids. We demonstrate that Pb²⁺ successfully targets oxygen-rich Ca²⁺ coordination sites of both C2 domains in SytI, a key regulator of neurotransmitter release. Our data provide structural and mechanistic insights into potential modes of Pb²⁺ toxicity and interference with Ca²⁺-regulated processes.

INTRODUCTION

Lead poisoning remains a pervasive public health problem, as illustrated by the recent outbreaks in the U.S. (Flint, Michigan) and abroad.^{1, 2} Lead exposure is especially detrimental in young children, resulting in serious neurodevelopmental and psychological

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disorders.^{3–5} The potency of Pb^{2+} ($[\text{Xe}]-4f^{14}5d^{10}6s^2$) stems from its ability to cross the blood-brain barrier⁶ and preferentially target Zn^{2+} and Ca^{2+} coordination sites of biological macromolecules.^{7–9} The ability of Pb^{2+} to mimic these essential divalent metal ions results in disruption of cellular signaling, ion transport, and calcium homeostasis.^{10–13}

The molecular mechanisms of Pb^{2+} neurotoxicity are not well understood. Several neuronal proteins associated with Ca^{2+} signaling have been implicated in Pb^{2+} toxicity (reviewed in⁸). Among them are the voltage-gated Ca^{2+} channels, where the putative mechanism is the blockage of Ca^{2+} currents due to Pb^{2+} interactions with ion selectivity filters.^{14, 15} Another example is the ligand-gated ionotropic N-methyl D-aspartate receptor (NMDAR),^{16, 17} where Pb^{2+} acts as an antagonist, partly through the interactions with the allosteric Zn^{2+} regulatory site¹⁸ in the extracellular domain of the receptor. An important class of Pb^{2+} targets are the intracellular Ca^{2+} -sensor proteins, such as Synaptotagmin I (SytI),¹⁹ Calmodulin (CaM),^{20, 21} and protein kinase C (PKC).^{22, 23}

While the proteins in question are quite distinct in their structure and function, one shared feature is the prevalence of oxygen donor ligands in their metal-ion coordination sites. The proposed NMDAR Pb^{2+} -binding site comprises the oxygens of aspartate and glutamate carboxylate groups, along with additional nitrogen ligands provided by histidine residues.²⁴ The selectivity filters of Ca^{2+} channels,^{14, 25} the EF hand motif of CaM,²¹ and the loop regions of the Ca^{2+} -dependent phospholipid-binding conserved homology 2 (C2) domains of SytI^{19, 26} and PKC²³ have all-oxygen metal-ion coordination sites capable of interactions with Pb^{2+} . The analysis of Pb^{2+} -bound protein structures in the PDB revealed that about 79% of the Pb^{2+} -coordinating ligands are oxygen atoms that belong to the sidechain carboxylate and backbone carbonyl moieties of proteins, in addition to surrounding water molecules.²⁷ The objective of this work was to determine what makes Pb^{2+} an effective competitor for oxygen-rich coordination sites in proteins, using SytI as a paradigm.

SytI is an integral membrane protein that serves as a Ca^{2+} -dependent trigger of synchronous neurotransmitter release.²⁸ The N-terminal segment of SytI is a transmembrane helical domain that anchors the protein to synaptic vesicle (Fig. 1a). The cytosolic C-terminal region comprises two Ca^{2+} -sensing C2 domains, C2A and C2B. These domains have tri-partite (C2A) and bi-partite (C2B) Ca^{2+} binding motifs that are believed to be targeted by Pb^{2+} with unknown stoichiometry.^{19, 26} The intrinsic Ca^{2+} affinities are pH-dependent and weak, ranging from 50 μM to >10 mM.^{29–31} Ca^{2+} binding generates a localized electropositive potential in the apical C2 loop region and thereby enables SytI to interact with presynaptic membranes and SNARE proteins (Fig. 1a).^{29, 32–36} The outcome is the exocytic membrane fusion with the concomitant release of neurotransmitters into the synaptic cleft.

In this work, we demonstrate that SytI has two high-affinity Pb^{2+} binding sites, one per C2 domain. These high-affinity interactions, combined with fast binding and slow dissociation, impart thermodynamic and kinetic advantage on Pb^{2+} compared to Ca^{2+} . Moreover, a single Pb^{2+} ion binding to either C2 domain has a profound inhibitory effect on subsequent Ca^{2+} binding, despite the existing coordination vacancies. Together, the inhibition of Ca^{2+} binding and previously known ability of Pb^{2+} to trigger membrane association of C2

domains^{19, 23, 37} provide a potential mechanism to explain the effect of Pb^{2+} on neurotransmitter release.

METHODS

Materials

The working solutions of metal ions were prepared in HPLC grade water or decalcified buffers using the following salts: Pb(II) acetate tri-hydrate (Sigma-Aldrich), standardized 1 M solution of Ca(II) chloride (Fluka Analytical), and Tb(III) chloride hexahydrate (Acros Organics). Prior to use, all buffers were treated with the ion-chelating resin Chelex 100 (Sigma-Aldrich) to remove trace divalent metals. Lipid components used in the phospholipid vesicle preparations: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). The quartz cuvettes used for the Tb^{3+} luminescence experiments were coated with Sigmacote® to avoid the protein adhesion to the walls. The cDNA of murine SytI was purchased from Open Biosystems (GE Life Sciences). All protein constructs were expressed and purified as described in the SI.

Crystallization, structure determination and refinement

The samples used for crystallization of SytI domains with Pb^{2+} contained: (i) 17 mg/mL C2A with 7 mM Pb(II) acetate, and (ii) 22 mg/mL C2B with 1.1 mM Pb(II) acetate in 20 mM MES buffer at pH 6.0. Screening for crystallization was carried out in automated manner by using the sitting drop vapor-diffusion method with an Art Robbins Instruments Phoenix system in the X-ray Crystallography Core Laboratory at UTHSCSA. Crystals for Pb^{2+} -bound C2A were obtained from Qiagen Classics II Suite condition #74 (0.2 M lithium sulfate, 0.1 M bis-tris pH 5.5, 25% polyethylene glycol 3350) at 4 °C. Although C2B was loaded with Pb^{2+} prior to crystallization, it was difficult to produce a Pb^{2+} -loaded C2B crystal as the metal was typically lost resulting in apo-C2B crystals. Crystals for Pb^{2+} -bound C2B were ultimately obtained from Microlytic MCSG-2 Suite condition #33 (0.2 M sodium fluoride, 20% polyethylene glycol 3350) at 22 °C. The crystals exhibited low occupancy Pb^{2+} -binding during refinement of the structure coordinates, so an additional crystal was soaked overnight in mother liquor containing 5 mM lead acetate. This technique was applied to promote complete Pb^{2+} -binding since the unsoaked crystal structure showed ambiguity in some of the electron density containing the binding site. The details of structure determination and refinement are given in the SI, along with the data collection and refinement statistics (Table S1). The refined coordinates of the Pb^{2+} complexes of C2A and C2B were deposited in the Protein Data Bank under accession codes 5vfe and 5vfg (5vff for partial Pb^{2+} occupancy), respectively. The analysis of metal-oxygen distances and the calculation of backbone r.m.s.d. from the previously published SytI structures (Tables S2-S5) was conducted using UCSF Chimera.³⁸

Isothermal Titration Calorimetry (ITC)

For ITC experiments, the C2A and C2B domains of SytI were extensively dialyzed against the large excess of decalcified ITC buffer (20 mM MES at pH 6.0 with 150 mM KCl). The filtered and degassed dialysis buffer was then used to prepare 50 μM C2A/C2B and 0.5 mM

Pb²⁺ working solutions. The measurements for the heat of binding were carried out in MicroCal iTC200 (Malvern Panalytical) instrument with 14 successive additions of Pb²⁺ stock solution (0.5 µl for the first injection and 3 µl for all subsequent injections) into the protein. The acquisition and analysis of the triplicates was done using Origin software; the data were fit into a single set-of-sites binding model.

Nuclear Magnetic Resonance (NMR) spectroscopy

NMR-detected Pb²⁺ binding to C2 domains—NMR-detected Pb²⁺ binding to [U-¹⁵N] enriched SytI C2 domains was monitored by acquiring series of ¹H-¹⁵N HSQC spectra at 25 °C on Bruker AVANCE III spectrometers operating at ¹H Larmor frequencies of 500 MHz (C2B) and 600 MHz (C2A). Protein concentration of 100 µM in decalcified 20 mM MES buffer (pH 6.0), 0.02% NaN₃, and 8% D₂O was used for all binding experiments. The Pb²⁺ concentrations were: 0, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.3, 1.6, 2.0, and 2.5 mM for C2A; and 0, 0.0125, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.8, 1.5, and 3.0 mM for C2B. The spectra were processed using NMRPipe³⁹ and analyzed using Sparky.⁴⁰ The chemical shift perturbation (CSP) due to M²⁺ binding, Δ , was calculated using the following equation:

$$\Delta = [\Delta\delta_H^2 + (0.152\Delta\delta_N)^2]^{1/2} \quad (1)$$

where δ_H and δ_N are residue-specific ¹H and ¹⁵N chemical shift differences between the apo and Pb²⁺ bound states of the proteins. Pb²⁺ binding curves for the second site of the respective domains were constructed by plotting Δ as a function of corrected Pb²⁺ concentration to take into account the partial occupancy of the first metal binding sites. The binding curves were globally fitted (12 C2A, and 9 C2B residues) with a single-site binding model:

$$\Delta = (\Delta_{\max}/2P_0)[(K_d + P_0 + L_0) - ((K_d + P_0 + L_0)^2 - 4P_0L_0)^{1/2}] \quad (2)$$

where Δ is the CSP value between the apo and Pb²⁺-bound state; $\Delta_{\max}$ is the maximum CSP value reached upon Site 2 saturation; and P_0 and L_0 are the total protein and Pb²⁺ concentrations, the latter corrected for Pb²⁺ populating Site 1.

ZZ exchange NMR spectroscopy—The kinetic parameters of Pb²⁺ binding to the high-affinity sites of [U-¹⁵N] enriched SytI C2 domains were obtained by acquiring a series of ZZ exchange experiments⁴¹ on the cryoprobe-equipped Bruker AVANCE III spectrometers operating at ¹H Larmor frequencies of 800 MHz (C2A) and 600 MHz (C2B). The data were collected at 4 different temperatures: 10, 15, 20, and 25 °C. The temperatures were calibrated using deuterated methanol. The protein samples (350 µM) were prepared in decalcified 20 mM MES buffer (pH 6.0), 150 mM KCl, 0.02% NaN₃, and 8% D₂O. Pb²⁺ was added to a concentration of 175 µM to generate approximately equal populations of the apo- and Pb²⁺-bound proteins. The samples were equilibrated overnight. The exchange between the two states, apo and Pb²⁺-bound, resulted in the transfer of longitudinal ¹⁵N

magnetization during the variable mixing time period, manifested as the build-up of the cross-peak intensities and decay of the auto-peak intensities. The respective build-up and decay of the cross-peak and auto-peak intensities for the well-resolved residues was quantified as a function of effective mixing times: 12.53, 17.53, 22.53, 27.53, 32.53, 37.53, and 42.53 ms for C2A and an additional point of 52.53 ms for C2B (Fig. S3). Effective mixing times, t_{mix} , were calculated as the duration of the mixing period plus 12.53 ms, which the time that ^{15}N magnetization was longitudinal during the other elements of the pulse sequence. The spectra were processed using NMRPipe³⁹ and analyzed using Sparky.⁴⁰ The analysis of the ZZ exchange data was conducted as described in the SI, following the formalism of Miloushev et al.⁴²

Detection of mixed $\text{Pb}^{2+}/\text{Ca}^{2+}$ C2 species—The formation of $\text{C2}\cdot\text{Pb1}\cdot(\text{Ca})_n$ complexes ($n=1,2$ for C2A and $n=1$ for C2B) was monitored at 25 °C on Bruker AVANCE III spectrometers operating at ^1H Larmor frequencies of 800 MHz (C2A) and 500 MHz (C2B). The C2 domains were first equilibrated with concentrations of Pb^{2+} sufficient to selectively populate first metal binding sites in a buffer solution composed of decalcified 20 mM MES (pH 6.0), 150 mM KCl, 0.02% NaN_3 , and 8% D_2O . The aliquots of Ca^{2+} solution prepared in the NMR buffer were added to the samples to achieve concentrations ranging from 100 μM to 40 mM. The spectral changes were monitored using ^{15}N - ^1H HSQC spectra.

Tb^{3+} luminescence and vesicle co-sedimentation experiments are described in the SI.

RESULTS AND DISCUSSION

Pb^{2+} targets Ca^{2+} Site 1 in both C2 domains of SytI

Previous studies of SytI suggested that Pb^{2+} binding site resides on the C2A domain.¹⁹ It was unclear to us from the structural viewpoint why C2A would be a preferred interaction site over C2B. To understand the structural basis of $\text{C2}\cdot\text{Pb}^{2+}$ interactions in SytI, we determined two high-resolution crystal structures of Pb^{2+} -complexed C2A and C2B domains. Both structures revealed the presence of a single Pb^{2+} ion that was refined at high occupancy: $\text{C2A}\cdot\text{Pb1}$ (1.4 Å, 5vfe) and $\text{C2B}\cdot\text{Pb1}$ (1.8 Å, 5vfg) (Table S1 and Fig. 1b). We found that the position of bound Pb^{2+} ion (Pb1) coincides with that of the first Ca^{2+} ion (subsequently referred to as Ca1, see Fig. 1e,f), as defined in previous structural studies of Ca^{2+} -complexed C2 domains. Comparison of Pb^{2+} -bound and apo structures showed that the most conformational changes due to Pb^{2+} -binding are in the apical loop regions, specifically loop 1 in C2A and loop 3 in C2B (Fig. 1b). Close inspection of the Pb^{2+} coordination sites revealed that these differences are due to the rotation of the coordinating aspartic acid sidechains towards the metal ion: Asp178 in C2A, and Asp365 and Asp303 in C2B (Figs. 1c,d). The conformational changes in the other regions of C2 domains are minor, as evidenced by the low r.m.s.d. values obtained from the comparative analysis of existing C2A and C2B structures (Tables S2-S3).

Pb^{2+} ions bound to the C2A and C2B domains have a coordination number (CN) of 8. All ligands are oxygen atoms donated by the aspartic acid sidechains, one backbone carbonyl group, and one (or two in case of C2A) water molecules (Figs. 1c,d). The ligands are distributed uniformly in the coordination sphere, indicating that the $6s^2$ lone pair of Pb^{2+} is

stereo-chemically inactive. The distribution of Pb-oxygen bond distances is narrow, ranging from 2.4 to 2.8 Å (Tables S4-S5). In coordination chemistry of Pb²⁺, uniform distribution of ligands and narrow range of Pb-ligand distances define a holodirected coordination geometry, which is favored in Pb²⁺ sites with high CN values and bulky ligands.⁴³

One notable difference between the Pb²⁺- and Ca²⁺-complexed C2A structures is a lack of coordination bond between Pb1 and the sidechain oxygens of Asp232, which points away from the metal ion binding site (Fig. 1e); in contrast, the Ca²⁺-Asp232 O61 coordination bond is present in the NMR structure of C2A (1byn).⁴⁴ In C2B, the coordination geometry of Ca1 and Pb1 is identical, which is also reflected in the similarity of the backbone conformation of the loop regions.

Each C2 domain has one tight and one weak Pb²⁺ site

C2A and C2B have tri- and bi-partite Ca²⁺-binding motifs, respectively. To determine how many sites Pb²⁺ populates in solution, we conducted NMR-detected binding experiments of Pb²⁺ to the C2 domains. The chemical shift changes of the N-H_N backbone groups proximal to the M²⁺ coordination centers revealed two distinct Pb²⁺ binding events in C2A (Fig. 2a) and C2B (Fig. 3a). The first Pb²⁺ binding event, which is “slow” on the NMR chemical shift timescale for the majority of residues and saturates at approximately stoichiometric concentrations of Pb²⁺, gives rise to two sets of cross-peaks that correspond to the apo C2 and the C2·Pb1 complex. The second binding event is “fast”, manifesting itself in the smooth cross-peak trajectories as a function of increasing total Pb²⁺ concentration. These data indicate the presence of two Pb²⁺ sites with distinct kinetics and thermodynamics of binding. We did not observe an appreciable population of Site 3 by Pb²⁺ in the C2A domain.

To determine the influence of Pb²⁺ binding on the C2 loop regions, we constructed the chemical shift perturbation (CSP) plots for the high and low Pb²⁺ concentration regimes (Fig. 2b and Fig. S1b). The low concentration regime mostly reflects protein response to binding event 1, while the high concentration regime reflects the response to binding event 2. The CSP plot of C2B shows that all three loop regions are affected by interactions with Pb²⁺, with the most changes caused by the first binding event (Fig. S1b). In C2A, the second binding event influences the residues of loop 3 more than those of loop 1 (Fig. 2b). Using this information in conjunction with the Ca²⁺-bound C2A structure, we can then assign the low-affinity Pb²⁺ site to Site 2 and high-affinity Pb²⁺ site to Site 1 in C2A, which is populated in the crystal structure of the Pb²⁺ complex.

We used the fast-exchange NMR data to construct the binding curves and obtain Pb²⁺ affinities to Site 2 of the C2 domains (see Fig. 2c and Fig. 3b for the Site 2 location). Global fitting of the binding curves produced K_d values of $330 \pm 10 \mu\text{M}$ (C2A, Fig. 2d) and $220 \pm 5 \mu\text{M}$ (C2B, Fig. S1c). The Ca²⁺ affinities for the same sites under identical buffer conditions are 1.6 mM (C2A) and 0.7–0.8 mM (C2B).³¹ This means that the affinity of Pb²⁺ to Site 2 exceeds that of Ca²⁺ by 5- and 3-fold in the C2A and C2B domains, respectively.

The slow exchange regime displayed by Pb²⁺ binding to Site 1 is generally unsuitable for the determination of binding affinities. We therefore conducted ITC experiments to obtain the dissociation constants (K_d) and thermodynamic parameters of Pb²⁺ binding to Site 1.

The K_d values of Pb1 are in the sub-micromolar range for both domains: $0.68 \pm 0.05 \mu\text{M}$ (C2A) and $0.47 \pm 0.1 \mu\text{M}$ (C2B), respectively (Fig. 3c). This represents 340-fold (C2A) and 1,400-fold (C2B) enhancement of Pb^{2+} affinities compared to those of Ca^{2+} under identical buffer conditions.³¹ The underlying thermodynamic basis of this enhancement is evident from the comparison of the enthalpic and entropic contributions to ΔG (Fig. 3d). Pb1 binding to C2 domains is significantly exothermic. Combined with the favorable entropic contribution, this leads to large negative ΔG values. In contrast, the enthalpic contribution to Ca1 binding is small (inset of Fig. 3d), with ΔG being dominated by the entropy term. Therefore, it is mostly the differences in binding enthalpies that are responsible for the differential affinities of Pb1 and Ca1. The positive entropy change for both metal ions indicates that the gain due to metal de-solvation⁴⁶ is sufficient to compensate for the loss of conformational flexibility of metal ion-coordinating ligands.

A comparative summary of the binding data illustrates the two main conclusions of our experiments (Fig. 3e). First, Pb^{2+} populates Sites 1 and 2 in solution, with Pb1 affinity exceeding that of Pb2 by ~500-fold; this property enabled us to selectively probe Pb^{2+} binding events using ITC and NMR. Second, Pb^{2+} affinities are higher than those of Ca^{2+} for both C2 domains. The enhancement of Pb^{2+} affinities compared to those of Ca^{2+} is significantly more pronounced for Site 1, which is the only site populated in the crystal structures of C2A and C2B.

Our conclusions regarding differential affinities of Pb^{2+} and Ca^{2+} to the C2 domains of SytI are further supported by the results of Tb^{3+} displacement experiments (Fig. 4). Tb^{3+} binds to the cytoplasmic region of SytI that contains both C2A and C2B domains (C2AB) with an apparent affinity of $2.5 \mu\text{M}$ (data not shown). When bound to C2AB, Tb^{3+} shows a strong luminescence signal due to FRET from the tryptophan sidechains. We prepared a fully Tb^{3+} -saturated C2AB and monitored the intensity changes of the strongest luminescence peak at 545 nm upon addition of Ca^{2+} and Pb^{2+} (Fig. 4a,b). It takes ~100-fold more Ca^{2+} than Pb^{2+} to achieve ~50% Tb^{3+} displacement from C2AB (Fig. 4c), clearly indicating the thermodynamic preference for Pb^{2+} over Ca^{2+} .

It is well established that the affinities of divalent metal ions to C2 domains significantly increase in the vicinity of anionic lipids, in what Falke coined as a “target-activated messenger affinity” (TAMA) mechanism.⁴⁷ The implication is that intrinsic metal-ion affinities that we measure in solution are 2–3 orders of magnitude lower than those at the membrane surface. This mechanism provides an explanation of why C2 domains, being intrinsically weak Ca^{2+} -binding modules in solution, are able to respond to low micromolar Ca^{2+} concentrations during the cell-signaling event. In the framework of the TAMA mechanism, the affinity of Pb^{2+} to Site 1 would be comparable to the bioavailable concentration of Pb^{2+} , which ranges from picomolar to nanomolar.⁴⁸ This would make Pb^{2+} binding feasible under physiological conditions. The role of Ca^{2+} binding to Site 1 in C2 domains is the initial weak recruitment of the protein complex to the membranes, with the subsequent population of remaining Ca^{2+} site(s) to ensure high-affinity interaction.⁴⁷ Consistent with these findings, we observed very weak interactions between the C2·Pb1 complexes and phosphatidylserine-containing LUVs (data not shown), but almost full membrane association of C2 under saturating Pb^{2+} conditions (Fig. S2). Based on the above

considerations, we conclude that: (i) Pb^{2+} is isofunctional to Ca^{2+} in its ability to support the C2-membrane interactions; and (ii) interactions of Ca^{2+} and Pb^{2+} with Site 1 of C2 domains will primarily determine the competitive behavior of these metal ions. We next sought to explore the properties of the C2·Pb1 complexes that – in addition to relative Ca1 and Pb1 affinities (Fig. 3e) – are most relevant for $\text{Pb}^{2+}/\text{Ca}^{2+}$ competition: the kinetics of Pb^{2+} binding to Site 1 and the formation of mixed metal ion C2 species.

Fast binding and slow dissociation of Pb^{2+} from Site 1

To obtain the kinetic information on Pb^{2+} binding to Site 1 of the C2A and C2B domains, we used the ZZ-exchange solution NMR spectroscopy. The method relies on the exchange of longitudinal magnetization between two C2 domain species: apo and single Pb^{2+} -bound, C2·Pb1 (Fig. 5a,b). The inter-conversion between apo C2 and C2·Pb1 gives rise to cross-peaks (inset of Fig. 5c,d). The time-dependence of the auto- and cross-peak intensities, expressed through the composite ratio Ξ ,⁴² contains information on the on- and off-rate constants for Pb^{2+} binding to Site 1 (Figs. S3–4 and Fig. 5c,d). The NMR data analysis produced the on-rate constants: $(6.8 \pm 0.4) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (C2A) and $(6.2 \pm 0.3) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (C2B) that are close to the diffusion limit of $6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at 25 °C.⁴⁹ Moreover, the Pb^{2+} k_{on} values are comparable to the k_{on} value previously reported for Ca1 binding to the C2A domain, $(3.9 \pm 0.8) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$.⁵⁰ In contrast to k_{on} , the Pb^{2+} k_{off} values of 45.5 (C2A) and 29 s^{-1} (C2B) are approximately two orders of magnitude smaller than the 2000–9500 s^{-1} range previously reported for Ca^{2+} .^{50–52} Therefore, the differential affinities of Ca^{2+} and Pb^{2+} to Site 1 are due to the differences in the off-rate constants.

The temperature-dependent kinetics data were further used to estimate the activation enthalpy H and activation entropy S for the forward and reverse reactions (Fig. 5e). Although the enthalpic barrier H_f for the C2A- Pb^{2+} association (9.3 kcal/mol) is larger than that of C2B (6.6 kcal/mol), the accompanying differences in S_f values produce essentially identical G_f values for C2A and C2B, 6.8 kcal/mol at 25 °C. This is only 1.8 kcal/mol larger than the theoretically predicted energy cost of ~5 kcal/mol required to transport a small molecule at the diffusion limit.⁴⁹ The negligible S_f for the C2B- Pb^{2+} association indicates that the gain in solvent entropy due to de-solvation of Pb^{2+} and protein binding region is offset by a loss of conformational flexibility of C2B in the transition state. This is in contrast to C2A, where the positive value of S_f suggests that conformational flexibility in the transition state is partially preserved. In the reverse (dissociation) direction, the activation parameters for the C2A and C2B are similar, with enthalpy and entropy terms contributing 80% and 20% to G_r , respectively. In aggregate, our data suggest that Pb^{2+} can act as both a thermodynamic and kinetic trap for the C2 domains and thereby effectively compete with Ca^{2+} for Site 1.

Pb^{2+} binding to Site 1 locks C2 domains in Ca^{2+} -insensitive state

One potential mechanism through which Ca^{2+} can possibly rescue⁵³ the Pb^{2+} -induced protein behavior is through the formation of mixed metal ion species, with Pb^{2+} populating Site 1 and Ca^{2+} populating Site(s) 2 and 3, respectively (Fig. 6a). To test if this could be the case in SytI, we prepared C2·Pb1 complexes and evaluated their Ca^{2+} -binding behavior in the Ca^{2+} concentration range from 0.1 to 40 mM, using solution NMR spectroscopy. The

NMR samples were prepared such that the populations of C2A·Pb1 and C2B·Pb1 complexes were the dominant species (~ 95 %), with negligible population of Site 2 by Pb²⁺. To our surprise, it took mM Ca²⁺ concentrations to detect noticeable shifts in the NMR spectra of C2·Pb1. Only at very high concentrations of Ca²⁺ (10–40 mM) did we observe a clear titratable Ca²⁺-dependent behavior of cross-peaks that belong to the loop residues (**Fig. 6b,c**; Fig S5).

This is in sharp contrast with the Ca²⁺-only binding data that showed full saturation of Sites 1 and 2 at ~10 mM Ca²⁺ (insets of Fig. 6b,c). The dissociation constants of Ca²⁺ from Site 2 of the C2A·Pb1 complexes, $K_{d,Pb1}(Ca2)$, obtained from the NMR data analysis are ~120 and ~49 mM for the C2A and C2B, respectively (Fig. 6d). Comparison of the $K_{d,Pb1}(Ca2)$ with previously reported $K_{d,Ca1}(Ca2)$ values³¹ indicates that population of Site 1 by Pb²⁺ reduces Ca²⁺ affinities to Site 2 by 60–70 fold. Moreover, the same pattern holds for the C2 domain from another protein, Protein Kinase Ca²³ (Fig. 6e). Therefore, for three C2 domains that share about ~40% pairwise sequence identity we observed the same pattern: binding of Pb²⁺ to the high-affinity Site 1 on C2 domains reduces the Ca²⁺ affinity to the remaining vacant sites.

In view of the modest structural changes caused by Pb²⁺ binding to the C2 domain, the most likely explanation of this behavior lies in the electronic properties of Pb²⁺ influencing Ca²⁺ affinity through the “bridging” ligands. Inspection of the Ca²⁺-complexed structures of C2A and C2B shows that in each domain there are two oxygen atoms that bridge metal ions in Sites 1 and 2: Oδ1(Asp172) and Oδ1(Asp232) in C2A, and Oδ1(Asp303) and Oδ1(Asp365) in C2B (Fig. S6a,b).^{44, 54} Pb²⁺ is a stronger Lewis acid than Ca²⁺, which is manifested in its higher electronegativity.^{55, 56} This implies that Pb-O bonds have a more covalent character than Ca-O bonds. A significant depletion of electron density of bridging ligands by Pb²⁺ at Site 1 would reduce their electron-donating abilities and result in the attenuation of Ca²⁺ affinities to Site 2. If we apply the same rationale to describe Ca²⁺ interactions with Site 3 of the C2A·Pb1 complex, then Ca²⁺ affinity should not be significantly affected because metal ions in Sites 1 and 3 do not share any oxygen ligands. Indeed, the $K_{d,Pb1}(Ca3)$ of 26 mM (Fig. S6,c) determined using our NMR data is not significantly different from the >10 mM estimate reported for the Ca²⁺-only system.³⁰ Another important outcome of Ca²⁺-binding experiments is that we did not observe any evidence of Pb²⁺ displacement from Site 1 even at > 250-fold Ca²⁺ excess, further confirming our prediction that Pb²⁺ can effectively compete with Ca²⁺ for Site 1.

CONCLUSIONS

We have characterized the structural, thermodynamic, and kinetic aspects of Pb²⁺ interactions with the C2 domains of SytI, a key regulator of the synaptic vesicle fusion and neurotransmitter release. We established that the Ca²⁺-binding Site 1 of the C2 domains is the most likely target of Pb²⁺ due to high affinity of the interactions. The slow dissociation kinetics of Pb²⁺ will increase the lifetime of the protein-Pb²⁺ complexes in the cell and thereby make Pb²⁺ a potent competitor of Ca²⁺. The most unexpected outcome of our study – the loss of Ca²⁺ sensitivity of the C2 domains when Pb²⁺ populates only a single high-affinity site – suggests possible mechanisms through which Pb²⁺, despite its low

bioavailability, can disrupt the function of Ca^{2+} -dependent proteins. For instance, the inhibition of Ca^{2+} -dependent synchronous release of neurotransmitters^{57–64} observed upon Pb^{2+} exposure could be attributed to the failure of Pb^{2+} -complexed SytI to sense elevated intracellular Ca^{2+} concentrations. In addition, the ability of Pb^{2+} to support the membrane interactions of SytI can explain the observed stimulatory effect of Pb^{2+} on Ca^{2+} -independent spontaneous release.^{57–64} Previously, Bouton et al¹⁹ demonstrated that Pb^{2+} is ~1000-fold more potent than Ca^{2+} in driving the membrane association of the cytoplasmic region containing both C2 domains of SytI. Combined with the results reported here, this offers an intriguing possibility that, in contrast to a full complement of Ca^{2+} ions, only one Pb^{2+} ion per C2 domain might be sufficient to drive the membrane interactions of SytI. Membrane-binding experiments on full-length SytI reconstituted into membrane-mimicking environment are required to address this question. Finally, our findings indicate that high-affinity interactions of Pb^{2+} with proteins are not limited to the thiol-rich coordination sites,^{65–69} but can also occur in the all-oxygen coordination environment provided by the C2 domains, the Ca^{2+} -sensing membrane-binding modules found in many signaling proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCE

1. Lo YC, Dooyema CA, Neri A, Durant J, Jefferies T, Medina-Marino A, de Ravello L, Thoroughman D, Davis L, Dankoli RS, Samson MY, Ibrahim LM, Okechukwu O, Umar-Tsafe NT, Dama AH and Brown MJ, Childhood lead poisoning associated with gold ore processing: a village-level investigation-Zamfara State, Nigeria, October-November 2010, Environ. Health Perspect, 2012, 120, 1450–1455. [PubMed: 22766030]
2. Hanna-Attisha M, LaChance J, Sadler RC and Champney Schnepf A, Elevated Blood Lead Levels in Children Associated With the Flint Drinking Water Crisis: A Spatial Analysis of Risk and Public Health Response, Am. J. Public Health, 2016, 106, 283–290. [PubMed: 26691115]
3. Canfield RL, Henderson CR, Jr, Cory-Slechta DA, Cox C, Jusko TA and Lanphear BP, Intellectual impairment in children with blood lead concentrations below 10 μg per deciliter, N. Engl. J. Med, 2003, 348, 1517–1526. [PubMed: 12700371]
4. Bellinger DC, Very low lead exposures and children's neurodevelopment, Curr. Opin. Pediatr, 2008, 20, 172–177. [PubMed: 18332714]
5. Gump BB, Dykas MJ, MacKenzie JA, Dumas AK, Hruska B, Ewart CK, Parsons PJ, Palmer CD and Bendinskas K, Background lead and mercury exposures: Psychological and behavioral problems in children, Environ. Res, 2017, 158, 576–582. [PubMed: 28715786]
6. Deane R and Bradbury M, Transport of Lead-203 at the Blood-Brain Barrier During Short Cerebrovascular Perfusion with Saline in the Rat, J. Neurochem, 1990, 54, 905–914. [PubMed: 2106011]
7. Godwin HA, The biological chemistry of lead, Curr. Opin. Chem. Biol, 2001, 5, 223–227. [PubMed: 11282351]

8. Gorkhali R, Huang K, Kirberger M and Yang JJ, Defining potential roles of Pb(2+) in neurotoxicity from a calciomics approach, *Metallomics*, 2016, 8, 563–578. [PubMed: 27108875]
9. Kirberger M, Pb (II) Disruption of Synaptic Activity Through Ca (II)-and Zn (II)-binding Proteins, *Neurotransmitter*, 2017, 4, 1–14.
10. Pounds JG, Wright R, Morrison D and Casciano DA, Effect of lead on calcium homeostasis in the isolated rat hepatocyte, *Toxicol. Appl. Pharmacol*, 1982, 63, 389–401. [PubMed: 7101300]
11. Bressler JP and Goldstein GW, Mechanisms of lead neurotoxicity, *Biochem. Pharmacol*, 1991, 41, 479–484. [PubMed: 1671748]
12. Lidsky TI and Schneider JS, Lead neurotoxicity in children: basic mechanisms and clinical correlates, *Brain*, 2003, 126, 5–19. [PubMed: 12477693]
13. Caito S, Lopes Ana Carolina BA, Paoliello Monica MB and Aschner M, *Journal*, 2017, 17.
14. Busselberg D, Evans ML, Rahmann H and Carpenter DO, Lead and zinc block a voltage-activated calcium channel of Aplysia neurons, *J. Neurophysiol*, 1991, 65, 786–795. [PubMed: 1646872]
15. Peng S, Hajela RK and Atchison WD, Characteristics of block by Pb²⁺ of function of human neuronal L-, N-, and R-type Ca²⁺ channels transiently expressed in human embryonic kidney 293 cells, *Mol. Pharmacol*, 2002, 62, 1418–1430. [PubMed: 12435810]
16. Neal AP and Guilarte TR, Molecular neurobiology of lead (Pb(2+)): effects on synaptic function, *Mol. Neurobiol*, 2010, 42, 151–160. [PubMed: 21042954]
17. Alkondon M, Costa AC, Radhakrishnan V, Aronstam RS and Albuquerque EX, Selective blockade of NMDA-activated channel currents may be implicated in learning deficits caused by lead, *FEBS Lett*, 1990, 261, 124–130. [PubMed: 1689669]
18. Gavazzo P, Zanardi I, Baranowska-Bosiacka I and Marchetti C, Molecular determinants of Pb²⁺ interaction with NMDA receptor channels, *Neurochem. Int*, 2008, 52, 329–337. [PubMed: 17706324]
19. Bouton CM, Frelin LP, Forde CE, Arnold Godwin H and Pevsner J, Synaptotagmin I is a molecular target for lead, *J. Neurochem*, 2001, 76, 1724–1735. [PubMed: 11259490]
20. Habermann E, Crowell K and Janicki P, Lead and other metals can substitute for Ca²⁺ in calmodulin, *Arch. Toxicol*, 1983, 54, 61–70. [PubMed: 6314931]
21. Kursula P and Majava V, A structural insight into lead neurotoxicity and calmodulin activation by heavy metals, *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun*, 2007, 63, 653–656.
22. Markovac J and Goldstein GW, Picomolar concentrations of lead stimulate brain protein kinase C, *Nature*, 1988, 334, 71–73. [PubMed: 3386747]
23. Morales KA, Lasagna M, Gribenko AV, Yoon Y, Reinhart GD, Lee JC, Cho W, Li P and Igumenova TI, Pb²⁺ as modulator of protein-membrane interactions, *J. Am. Chem. Soc*, 2011, 133, 10599–10611. [PubMed: 21615172]
24. Romero-Hernandez A, Simorowski N, Karakas E and Furukawa H, Molecular Basis for Subtype Specificity and High-Affinity Zinc Inhibition in the GluN1-GluN2A NMDA Receptor Amino-Terminal Domain, *Neuron*, 2016, 92, 1324–1336. [PubMed: 27916457]
25. Tang L, Gamal El-Din TM, Payandeh J, Martinez GQ, Heard TM, Scheuer T, Zheng N and Catterall WA, Structural basis for Ca²⁺ selectivity of a voltage-gated calcium channel, *Nature*, 2014, 505, 56–61. [PubMed: 24270805]
26. van Severen MC, Piquemal JP and Parisel O, Lead Substitution in Synaptotagmin: A Case Study, *The Journal of Physical Chemistry B*, 2010, 114, 4005–4009. [PubMed: 20192256]
27. Kirberger M and Yang JJ, Structural differences between Pb²⁺- and Ca²⁺-binding sites in proteins: implications with respect to toxicity, *J. Inorg. Biochem*, 2008, 102, 1901–1909. [PubMed: 18684507]
28. Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF and Sudhof TC, Synaptotagmin I: a major Ca²⁺ sensor for transmitter release at a central synapse, *Cell*, 1994, 79, 717–727. [PubMed: 7954835]
29. Fernandez I, Arac D, Ubach J, Gerber SH, Shin O, Gao Y, Anderson RG, Sudhof TC and Rizo J, Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine, *Neuron*, 2001, 32, 1057–1069. [PubMed: 11754837]

30. Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C and Sudhof TC, Synaptotagmin I functions as a calcium regulator of release probability, *Nature*, 2001, 410, 41–49. [PubMed: 11242035]
31. Katti S, Nyenhuis SB, Her B, Srivastava AK, Taylor AB, Hart PJ, Cafiso DS and Igumenova TI, Non-Native Metal Ion Reveals the Role of Electrostatics in Synaptotagmin I-Membrane Interactions, *Biochemistry*, 2017, 56, 3283–3295. [PubMed: 28574251]
32. Zhang X, Rizo J and Sudhof TC, Mechanism of phospholipid binding by the C2A-domain of synaptotagmin I, *Biochemistry*, 1998, 37, 12395–12403. [PubMed: 9730811]
33. Bai J, Wang P and Chapman ER, C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, 99, 1665–1670. [PubMed: 11805296]
34. Shao X, Li C, Fernandez I, Zhang X, Sudhof TC and Rizo J, Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch, *Neuron*, 1997, 18, 133–142. [PubMed: 9010211]
35. Pang ZP, Shin OH, Meyer AC, Rosenmund C and Sudhof TC, A gain-of-function mutation in synaptotagmin-1 reveals a critical role of Ca2+-dependent soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex binding in synaptic exocytosis, *J. Neurosci.*, 2006, 26, 12556–12565. [PubMed: 17135417]
36. Zhou Q, Lai Y, Bacaj T, Zhao M, Lyubimov AY, Uervirojnangkoorn M, Zeldin OB, Brewster AS, Sauter NK, Cohen AE, Soltis SM, Alonso-Mori R, Chollet M, Lemke HT, Pfuetzner RA, Choi UB, Weis WI, Diao J, Sudhof TC and Brunger AT, Architecture of the synaptotagmin-SNARE machinery for neuronal exocytosis, *Nature*, 2015, 525, 62–67. [PubMed: 26280336]
37. Morales KA and Igumenova TI, Synergistic Effect of Pb2+ and Phosphatidylinositol 4, 5-Bisphosphate on C2 Domain–Membrane Interactions, *Biochemistry*, 2012, 51, 3349–3360. [PubMed: 22475207]
38. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC and Ferrin TE, UCSF Chimera--a visualization system for exploratory research and analysis, *J. Comput. Chem.*, 2004, 25, 1605–1612. [PubMed: 15264254]
39. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J and Bax A, NMRPipe: a multidimensional spectral processing system based on UNIX pipes, *J. Biomol. NMR*, 1995, 6, 277–293. [PubMed: 8520220]
40. Lee W, Tonelli M and Markley JL, NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy, *Bioinformatics*, 2015, 31, 1325–1327. [PubMed: 25505092]
41. Farrow NA, Zhang O, Forman-Kay JD and Kay LE, A heteronuclear correlation experiment for simultaneous determination of 15N longitudinal decay and chemical exchange rates of systems in slow equilibrium, *J. Biomol. NMR*, 1994, 4, 727–734. [PubMed: 7919956]
42. Miloshev VZ, Bahna F, Ciatto C, Ahlsen G, Honig B, Shapiro L and Palmer III AG, Dynamic properties of a type II cadherin adhesive domain: implications for the mechanism of strand-swapping of classical cadherins, *Structure*, 2008, 16, 1195–1205. [PubMed: 18682221]
43. Shimoni-Livny L, Glusker JP and Bock CW, Lone Pair Functionality in Divalent Lead Compounds, *Inorg. Chem.*, 1998, 37, 1853–1867.
44. Shao X, Fernandez I, Sudhof TC and Rizo J, Solution structures of the Ca2+-free and Ca2+-bound C2A domain of synaptotagmin I: does Ca2+ induce a conformational change?, *Biochemistry*, 1998, 37, 16106–16115. [PubMed: 9819203]
45. Evans CS, He Z, Bai H, Lou X, Jeggle P, Sutton RB, Edwardson JM and Chapman ER, Functional analysis of the interface between the tandem C2 domains of synaptotagmin-1, *Mol. Biol. Cell*, 2016, 27, 979–989. [PubMed: 26792839]
46. Gifford JL, Walsh MP and Vogel HJ, Structures and metal-ion-binding properties of the Ca2+-binding helix–loop–helix EF-hand motifs, *Biochem. J.*, 2007, 405, 199–221. [PubMed: 17590154]
47. Corbin JA, Evans JH, Landgraf KE and Falke JJ, Mechanism of specific membrane targeting by C2 domains: localized pools of target lipids enhance Ca2+ affinity, *Biochemistry*, 2007, 46, 4322–4336. [PubMed: 17367165]
48. Claudio ES, Godwin HA and Magyar JS, in *Prog. Inorg. Chem.*, John Wiley & Sons, Inc., 2003, DOI: 10.1002/0471267287.ch1, pp. 1–144.

49. van Holde K, A hypothesis concerning diffusion-limited protein–ligand interactions, *Biophys. Chem*, 2002, 101, 249–254. [PubMed: 12488005]
50. Millet O, Bernado P, Garcia J, Rizo J and Pons M, NMR measurement of the off rate from the first calcium-binding site of the synaptotagmin I C2A domain, *FEBS Lett*, 2002, 516, 93–96. [PubMed: 11959110]
51. Bollmann JH, Sakmann B and Borst JG, Calcium sensitivity of glutamate release in a calyx-type terminal, *Science*, 2000, 289, 953–957. [PubMed: 10937999]
52. Schneggenburger R and Neher E, Intracellular calcium dependence of transmitter release rates at a fast central synapse, *Nature*, 2000, 406, 889–893. [PubMed: 10972290]
53. Sun X, Tian X, Tomsig JL and Suszkiw JB, Analysis of differential effects of Pb²⁺ on protein kinase C isozymes, *Toxicol. Appl. Pharmacol*, 1999, 156, 40–45. [PubMed: 10101097]
54. Cheng Y, Sequeira SM, Malinina L, Tereshko V, Sollner TH and Patel DJ, Crystallographic identification of Ca²⁺ and Sr²⁺ coordination sites in synaptotagmin I C2B domain, *Protein Sci*, 2004, 13, 2665–2672. [PubMed: 15340165]
55. Cárdenas C and Ayers PW, How reliable is the hard–soft acid–base principle? An assessment from numerical simulations of electron transfer energies, *Phys. Chem. Chem. Phys*, 2013, 15, 13959–13968. [PubMed: 23846548]
56. Li K, Li M and Xue D, Solution-phase electronegativity scale: Insight into the chemical behaviors of metal ions in solution, *The Journal of Physical Chemistry A*, 2012, 116, 4192–4198. [PubMed: 22468570]
57. Manalis RS and Cooper GP, Letter: Presynaptic and postsynaptic effects of lead at the frog neuromuscular junction, *Nature*, 1973, 243, 354–356. [PubMed: 4542814]
58. Carroll PT, Silbergeld EK and Goldberg AM, Alteration of central cholinergic function by chronic lead acetate exposure, *Biochem. Pharmacol*, 1977, 26, 397–402. [PubMed: 849332]
59. Minnema DJ, Greenland RD and Michaelson IA, Effect of in vitro inorganic lead on dopamine release from superfused rat striatal synaptosomes, *Toxicol. Appl. Pharmacol*, 1986, 84, 400–411. [PubMed: 3715885]
60. Minnema DJ, Michaelson IA and Cooper GP, Calcium efflux and neurotransmitter release from rat hippocampal synaptosomes exposed to lead, *Toxicol. Appl. Pharmacol*, 1988, 92, 351–357. [PubMed: 2895506]
61. Lasley SM and Gilbert ME, Presynaptic glutamatergic function in dentate gyrus in vivo is diminished by chronic exposure to inorganic lead, *Brain Res*, 1996, 736, 125–134. [PubMed: 8930317]
62. Struzynska L and Rafalowska U, The effect of lead on dopamine, GABA and histidine spontaneous and KCl-dependent releases from rat brain synaptosomes, *Acta Neurobiol. Exp. (Wars.)*, 1994, 54, 201–207. [PubMed: 7817835]
63. Antonio MT and Leret ML, Study of the neurochemical alterations produced in discrete brain areas by perinatal low-level lead exposure, *Life Sci*, 2000, 67, 635–642. [PubMed: 12659169]
64. Lasley SM and Gilbert ME, Rat hippocampal glutamate and GABA release exhibit biphasic effects as a function of chronic lead exposure level, *Toxicol. Sci*, 2002, 66, 139–147. [PubMed: 11861981]
65. Simons TJ, The affinity of human erythrocyte porphobilinogen synthase for Zn²⁺ and Pb²⁺, *Eur. J. Biochem*, 1995, 234, 178–183. [PubMed: 8529638]
66. Erskine PT, Senior N, Awan S, Lambert R, Lewis G, Tickle IJ, Sarwar M, Spencer P, Thomas P, Warren MJ, Shoolingin-Jordan PM, Wood SP and Cooper JB, X-ray structure of 5-aminolaevulinate dehydratase, a hybrid aldolase, *Nat. Struct. Biol*, 1997, 4, 1025–1031. [PubMed: 9406553]
67. Magyar JS, Weng TC, Stern CM, Dye DF, Rous BW, Payne JC, Bridgewater BM, Mijovilovich A, Parkin G, Zaleski JM, Penner-Hahn JE and Godwin HA, Reexamination of lead(II) coordination preferences in sulfur-rich sites: implications for a critical mechanism of lead poisoning, *J. Am. Chem. Soc*, 2005, 127, 9495–9505. [PubMed: 15984876]
68. Neupane KP and Pecoraro VL, Probing a homoleptic PbS₃ coordination environment in a designed peptide using ²⁰⁷Pb NMR spectroscopy: implications for understanding the molecular basis of lead toxicity, *Angew. Chem. Int. Ed. Engl*, 2010, 49, 8177–8180. [PubMed: 20859984]

69. Ordemann JM and Austin RN, Lead neurotoxicity: exploring the potential impact of lead substitution in zinc-finger proteins on mental health, *Metallomics*, 2016, 8, 579–588. [PubMed: 26745006]

Figure 1. Structural analysis of Pb^{2+} -SytI interactions.

(a) SytI is a Ca^{2+} -dependent trigger of exocytic membrane fusion. Ca^{2+} binding to C2A and C2B domains drives their interaction with anionic phospholipids of the presynaptic membrane, PtdSer and PtdIns(4,5) P_2 . (b) Crystal structures of Pb^{2+} -complexed C2 domains reveal a single bound Pb^{2+} ion (sienna). Backbone superposition of Pb^{2+} -complexed (C2A, purple and C2B, blue) and apo C2 domains (gray) illustrates the extent of conformational changes in the backbone of loop regions. The PDB identifiers are: 5vfe (C2A·Pb1), 4wee (apo C2A), 5vfg (C2B·Pb1), and 5ccj (apo C2B). (c,d) Octa-coordinated geometry of C2-bound Pb^{2+} . The sidechain carbons and coordinating oxygens in the Pb^{2+} -complexed structures are shown in green and red, respectively. All ligands are oxygen atoms donated by protein and water. Pb^{2+} binding is accompanied by the conformational rearrangement of several coordinating residues that are shown in boldface. (e,f) Comparison of metal-ion coordination sites in Pb^{2+} - and Ca^{2+} -complexed C2A (1byn/NMR) and C2B (1tjx) domains. Pb^{2+} and Ca^{2+} are represented with sienna and gray spheres, respectively. The coordination geometry of Ca^{2+} is shown with dashed lines. Only Ca1 and protein ligands are shown for clarity.

Figure 2. C2A domain binds two Pb^{2+} ions in solution.

(a) Expansions of the C2A ^{15}N - ^1H HSQC region for Pb^{2+} concentrations ranging from 0 to 2.5 mM. Peak displacements due to binding Pb1 (1, slow exchange) and Pb2 (2, fast exchange) are shown with arrows. (b) C2A chemical shift perturbation plot constructed for the low- (< 0.125 mM) and high- (> 0.125 mM) concentration regimes of Pb^{2+} . (c) Loop region of the C2A showing the location of Ca^{2+} sites 1–3, along with residues whose ^{15}N - ^1H cross-peaks are labelled in (a) and/or used in (d). (d) NMR-detected binding curves constructed for the low-affinity Pb^{2+} Site 2. Solid lines represent the global fit that produced the K_d of $330 \pm 10 \mu\text{M}$.

Figure 3. C2 domains bind two Pb^{2+} ions in solution, with differential affinities.

(a) Expansions of C2B ^{15}N - 1H HSQC region for Pb^{2+} concentrations ranging from 0 to 3 mM. Peak displacements due to binding Pb1 (1, slow exchange) and Pb2 (2, fast exchange) are shown with arrows. (b) Loop region of C2B showing the location of Ca^{2+} sites 1 and 2 that are populated by Pb^{2+} in solution. (c) ITC profiles for Pb1 association with C2A (top) and C2B (bottom), and the respective dissociation constants. (d) Thermodynamic parameters of Pb1 binding to C2 domains. The inset shows Ca1 data reported in the previous study.⁴⁵ (e) Comparison of Pb^{2+} and Ca^{2+} affinities to sites 1 and 2 measured under identical conditions.

Figure 4. Pb²⁺ is more potent than Ca²⁺ in displacing Tb³⁺ from the SytI C2AB region. The most intense Tb³⁺ emission peak at 545 nm (⁵D₄ to ⁷F₅ transition) is shown as a function of increasing Ca²⁺ (a) and Pb²⁺ (b) concentrations. The decrease in luminescence is indicative of the displacement of Tb³⁺ from the protein by Ca²⁺ and Pb²⁺. (c) Intensity decrease at 545 nm plotted as a function of M²⁺ (M=Ca, Pb).

Figure 5. Kinetics of Pb^{2+} binding to Site 1 probed using ZZ exchange NMR spectroscopy.

Slow exchange behavior between the apo and C2·Pb1 forms is illustrated using expansions of ^{15}N - 1H HSQC spectra of C2A (**a**) and C2B (**b**) domains. Representative ZZ exchange data, showing the time dependence of composite ratio Ξ at two different temperatures for Gly175 in C2A (**c**) and Thr334 in C2B (**d**). (**e**) Eyring plots for the temperature range (10–25 °C) accessible to ZZ exchange spectroscopy in this kinetic regime. The values of H and S were determined from the linear fit, which is shown with a dashed line.

Figure 6. Pb^{2+} binding to Site 1 inhibits Ca^{2+} binding to the remaining coordination vacancies. (a) Location of metal ion coordination vacancies relative to Pb1 in the loop regions of C2A and C2B. (b,c) Expansions of the ^{15}N - ^1H HSQC spectra showing Ca^{2+} binding behavior of Gly175 in C2A-Pb1 (b) and K375 in C2B-Pb1 (c). The C2 states are: apo (gray), C2-Pb1 (sienna), and C2-Ca1/Ca2 (green), the latter representing C2 domains at intermediate Ca^{2+} concentrations. The insets show cross-peak trajectories obtained in Ca^{2+} -only binding experiments. While not even 40 mM Ca^{2+} is sufficient to saturate Site 2 of C2-Pb1 complexes, Site 2 saturates at <10 mM Ca^{2+} in the absence of pre-bound Pb^{2+} . (d) Representative Ca^{2+} binding curves constructed for the C2-Pb1 complexes. Solid lines represent a global fit to the single-site binding equation that produced estimates of the dissociation constants. (e) Comparison of Ca^{2+} dissociation constants from Site 2 of C2A and C2B, when Site 1 is occupied by Ca^{2+} (gray) and Pb^{2+} (black).